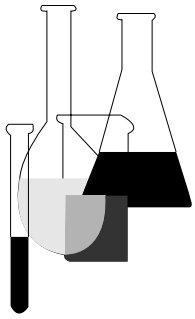
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–202
August 1998

Health Effects Test Guidelines

OPPTS 870.3250
90-Day Dermal Toxicity



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http://www.epa.gov/epahome/research.htm) under the heading ''Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines.''

OPPTS 870.3250 90-Day dermal toxicity.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline are 40 CFR 798.2250 Dermal Toxicity; OPP 82–3 90–Day Dermal (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals) EPA report 540/09–82–025, 1982; and OECD 411 Subchronic Dermal Toxicity: 90–Day.

(b) **Purpose.** In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic dermal study has been designed to permit the determination of the no-observed-effect level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. This study is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). Extrapolation from the results of this study to humans is valid only to a limited degree. It can, however, provide useful information on the degree of percutaneous absorption, target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(c) **Definitions.** The definitions in section 3 of the Toxic Substance Control Act (TSCA) and the definitions in 40 CFR Part 792--Good Laboratory Practice Standards apply to this test guideline. The following definitions also apply to this test guideline.

Cumulative toxicity is the adverse effect of repeated doses occurring as a result of prolonged action or increased concentration of the administered test substance or its metabolites in susceptible tissues.

Dose in a subchronic dermal study is the amount of test substance applied daily to the skin for 90 days. Dose is expressed as weight of the test substance (grams, milligrams), per unit body weight of test animal (milligrams per kilogram), or as weight of the test substance per unit of surface area (milligrams per square centimeter) per day.

No-observed-effect level (NOEL) is the maximum dose used in a study which produces no adverse effects. The NOEL is expressed in terms of the weight of a test substance given daily per unit weight of test animal (milligrams per kilogram per day).

Subchronic dermal toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the dermal route for a part of the test animal’s life span.

Target organ is any organ of a test animal showing evidence of an effect induced by a test substance.

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this guideline, produces no observable toxic effects or if toxic effects would not be expected based upon data on structurally related compounds, a full study using three dose levels might not be necessary.

(e) **Test procedures**—(1) **Animal selection**—(i) **Species and strain.** A mammalian species should be used for testing. The rat, rabbit, or guinea pig may be used. Commonly used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification/reasoning for their selection. When a subchronic dermal study is conducted as a preliminary to a chronic dermal study, the same species and strain should be used in both studies.

(ii) **Age/weight.** (A) Testing should be started with young healthy animals as soon as possible after weaning and acclimatization.

(B) Dosing should generally begin in guinea pigs between 5–6 weeks of age, in rats between 8–9 weeks of age, and in rabbits at least 12 weeks old.

(C) At the commencement of the study, the weight variation of animals used should be within 20 percent of the mean weight for each sex.

(iii) **Sex.** Equal numbers of animals of each sex with healthy skin should be used at each dose level. The females should be nulliparous and nonpregnant except for specially designed studies.

(iv) **Numbers.** (A) At least 20 animals (10 animals per sex) should be used at each dose level.

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before completion of the study.

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required.

(D) Each animal should be assigned a unique identification number. Dead animals, their preserved organs and tissues, and microscopic slides should be identified by reference to the animal's unique number.

(v) **Husbandry.** (A) Animals should be housed in individual cages.

(B) The temperature of the experimental animal rooms should be at 22 ± 3 °C

(C) The relative humidity of the experimental animal rooms should be 50 ± 20 percent.

(D) Where lighting is artificial, the sequence should be 12 hours light/ 12 hours dark.

(E) Control and test animals should be fed from the same batch and lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested and for impurities that might influence the outcome of the test. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

(F) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine. An acclimation period of at least five days is recommended.

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, the vehicle should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance. It is recommended that, whenever possible, the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then solution of other vehicles.

(ii) One lot of the test substance should be used, if possible, throughout the duration of the study, and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be a characterization of the test substance, including the purity of the test compound and if technically feasible, the name and quantities of unknown contaminants and impurities.

(iii) If the test substance is dissolved or suspended in a vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study. Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study. Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation, and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) **Control groups.** A concurrent control group is required. This group should be an untreated or sham-treated control group or, if a vehicle is used in the application of the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or not available, both untreated/sham-treated and vehicle control groups are required.

(4) **Satellite group.** A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days. In addition a control group of 20 animals (10 animals per sex) should be added to the satellite study.

(5) **Dose levels and dose selection.** (i) In subchronic toxicity tests, it is desirable to determine a dose-response relationship as well as a NOEL. Therefore, at least three dose levels plus a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest dose level) group should be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

(ii) The highest dose level should elicit signs of toxicity but not produce severe skin irritation or an incidence of fatality which would prevent a meaningful evaluation. If application of the test substance produces severe skin irritation, the concentration may be reduced, although this may result in a reduction in, or absence of, other toxic effects at the high dose level. If the skin has been badly damaged early in the study, it may be necessary to terminate the study and undertake a new one at lower concentrations.

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects.

(iv) The lowest dose level should not produce any evidence of toxic effects.

(6) **Preparation of animal skin.** Shortly before testing, fur should be clipped from not less than 10 percent of the body surface area for application of the test substance. In order to dose approximately 10 percent of the body surface, the area starting at the scapulae (shoulders) to the wing of the ileum (hipbone) and half way down the flank on each side of the animal should be shaved. Shaving should be carried out approximately 24 hours before dosing. Repeated clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care should be taken to avoid abrading the skin which could alter its permeability.

(7) **Preparation of test substance.** (i) Liquid test substances are generally used undiluted, except as indicated in paragraph (e)(5)(ii) of this guideline.

(ii) Solids should be pulverized when possible. The substance should be moistened sufficiently with water or, when necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence

of the vehicle on toxicity of, and penetration of the skin by, the test substance should be taken into account.

(iii) The volume of application should be kept constant, e.g., less than 300 μL for the rat; different concentrations of test solution should be prepared for different dose levels.

(8) **Administration of test substance.** (i) The duration of exposure should be at least for 90 days.

(ii) Ideally, the animals should be treated with test substance for at least 6 h/day on a 7-day per week basis. However, based on practical considerations, application on a 5-day per week basis is acceptable. Dosing should be conducted at approximately the same time each day.

(iii) The test substance should be applied uniformly over the treatment site.

(iv) The surface area covered may be less for highly toxic substances. As much of the area should be covered with as thin and uniform a film as possible.

(v) During the exposure period, the test substance should be held in contact with the skin with a porous gauze dressing (less than or equal to 8 ply). The test site should be further covered with nonirritating tape to retain the gauze dressing and the test substance and to ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not recommended. The test substance may be wiped from the skin after the six-hour exposure period to prevent ingestion.

(9) **Observation of animals.** (i) Observations should be made at least twice each day for morbidity and mortality. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals). General clinical observations should be made at least once a day, preferably at the same time each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animal should be recorded.

(ii) A careful clinical examination should be made at least once prior to the initiation of treatment (to allow for within subject comparisons) and once weekly during treatment in all animals. These observations should be made outside the home cage, preferably in a standard arena, and at similar times on each occasion. Effort should be made to ensure that variations in the observation conditions are minimal. Observations should be detailed and carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence

of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backwards) should be recorded.

(iii) Once, near the end of the exposure period and in any case not earlier than in week 11, assessment of motor activity, grip strength, and sensory reactivity to stimuli of different types (e.g., visual, auditory, and proprioceptive stimuli) should be conducted. Further details of the procedures that could be followed are described in the references listed under paragraphs (h)(1), (h)(3), (h)(4), (h)(5), (h)(6), and (h)(9) of this guideline.

(iv) Functional observations conducted towards the end of the study may be omitted when data on functional observations are available from other studies and the daily clinical observations did not reveal any functional deficits.

(v) Exceptionally, functional observations may be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with functional test performance.

(vi) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death.

(vii) Food consumption should also be determined weekly if abnormal body weight changes are observed.

(viii) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible.

(ix) At termination, all survivors in the control and treatment groups should be sacrificed.

(10) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group. The hematology and clinical chemistry parameters should be examined at terminal sacrifice at the end of the study. Overnight fasting of the animals prior to blood sampling is recommended. Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures.

(i) Hematology. The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time or activated partial thromboplastin time.

(ii) Clinical chemistry. (A) Parameters which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity.

(B) The recommended clinical chemistry determinations are potassium, sodium, glucose, total cholesterol, urea nitrogen, creatinine, total protein and albumin. More than 2 hepatic enzymes, (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, or gamma glutamyl transpeptidase) should also be measured. Measurements of additional enzymes (of hepatic or other origin) and bile acids, may also be useful.

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated.

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include calcium, phosphorus, fasting triglycerides, hormones, methemoglobin, and cholinesterases.

(iii) Optionally, the following urinalysis determinations could be performed during the last week of the study using timed urine volume collection: appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

(11) **Ophthalmological examination.** Using an ophthalmoscope or an equivalent device, ophthalmological examinations should be made on all animals prior to the administration of the test substance and on all high dose and control groups at termination. If changes in the eyes are detected, all animals in the other dose groups should be examined.

(12) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

(ii) The liver, brain, kidneys, spleen, adrenals, testes, epididymides, uterus, ovaries, thymus and heart should be trimmed and weighed wet, as soon as possible after dissection.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder (when present) .

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons), pituitary, peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic and lumbar), eyes (retina, optic nerve).

(C) Glandular system—adrenals, parathyroid, thyroid.

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose.

(E) Cardiovascular/Hematopoietic system—aorta, heart, bone marrow (and/or fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen, thymus.

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland

(G) Other—all gross lesions and masses, skin (both treated and adjacent untreated areas).

(13) **Histopathology.** (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed under paragraph (e)(12)(iii) of this guideline, of all animals in the control and high dose groups and all animals that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(D) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing toxic effects in the treated groups.

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) An attempt should be made to correlate gross observations with microscopic findings.

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming.

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group, number of animals at the start of the test, the number of animals showing lesions,

the types of lesions and the percentage of animals displaying each type of lesion.

(ii) When applicable, all observed results, qualitative and quantitative, should be evaluated by an appropriate and generally acceptable statistical method. Any, generally accepted statistical method should be used; the statistical methods including significance criteria should be selected during the design of the study.

(2) **Evaluation of study results.** The findings of a subchronic dermal toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of toxic effects and the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance, the incidence and severity of abnormalities including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effect on mortality, and any other general or specific toxic effects. A properly conducted 90-day subchronic dermal study should provide information on the effects of repeated application of a substance and a satisfactory estimation of a NOEL. It also can indicate the need for an additional longer-term study and provide information on the selection of dose levels.

(3) **Test report.** In addition to reporting requirements specified under EPA Good Laboratory Practice Standards at 40 CFR part 792, subpart J and 40 CFR part 160, and the OECD principles of GLP (ISBN 92-64-12367-9), the following specific information should be reported:

(i) Test substance characterization should include:

(A) Chemical identification.

(B) Lot or batch numbers.

(C) Physical properties.

(D) Purity/impurities.

(ii) Identification and composition of any vehicle if used.

(iii) Test system should contain data on:

(A) Species and strain of animals used and rationale for selection if other than that recommended.

(B) Age including body weight data and sex.

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods.

(D) Identification of animal diet.

(E) Acclimation period.

(iv) Test procedure should include the following data:

(A) Method of randomization used.

(B) Full description of experimental design and procedure.

(C) Dose regime including levels, method, and volume.

(v) Test results should include:

(A) Group animal data. Tabulation of toxic response data by species, strain, sex and exposure level for:

(*1*) Number of animals exposed.

(*2*) Number of animals showing signs of toxicity.

(*3*) Number of animals dying.

(B) Individual animal data. Data should be presented as summary (group mean) as well as for individual animals.

(*1*) Date of death during the study or whether animals survived to termination.

(*2*) Date of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data.

(*4*) Feed consumption data, when collected.

(*5*) Results of ophthalmological examination.

(*6*) Results of hematological tests performed.

(*7*) Results of clinical chemistry tests performed.

(*8*) Results of urinalysis, when performed.

(*9*) Results of observations made.

(*10*) Necropsy findings, including absolute and relative (to body weight) organ weight data.

(*11*) Detailed description of all histopathological findings.

(*12*) Statistical treatment of results, where appropriate.

(g) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study must be conducted in compliance with GLP regulations.

(h) **References.** The following references should be consulted for background information on this test guideline:

(1) Crofton K.M., Howard J.L., Moser V.C., Gill M.W., Leiter L.W., Tilson H.A., MacPhail, R.C. Interlaboratory Comparison of Motor Activity Experiments: Implication for Neurotoxicological Assessments. *Neurotoxicol. Teratol.* 13, 599-609. (1991)

(2) Draize, J.H. Dermal toxicity. Appraisal of Chemicals in Food, Drugs and Cosmetics. The Association of Food and Drug Officials of the United States (1959) 3rd printing 1975. pp. 46-59.

(3) Gad S.C. A Neuromuscular Screen for Use in Industrial Toxicology. *Journal of Toxicology and Environmental Health*. 9, 691-704. (1982)

(4) International Programme on Chemical Safety. Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals. Environmental Health Criteria Document No. 60. (1986)

(5) Meyer O.A., Tilson H.A., Byrd W.C., Riley M.T. A Method for the Routine Assessment of Fore- and Hind-Limb Grip Strength of Rats and Mice. *Neurobehav. Toxicol.* 1, 233-236. (1979)

(6) Moser V.C., McDaniel K.M., Phillips P.M. Rat Strain and Stock Comparisons using a Functional Observational Battery: Baseline Values and Effects of Amitraz. *Toxicol. Appl. Pharmacol.* 108, 267-283 (1991)

(7) National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances. A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(8) Organization for Economic Cooperation and Development. Guidelines for Testing of Chemicals, Section 4-Health Effects, Part 411 Subchronic Toxicity Studies, Paris, 1981.

(9) Tupper, D.E., Wallace R.B. Utility of the Neurologic Examination in Rats. Acta. *Neurobiol. Exp.* 40, 999-1003 (1980).

(10) United States Environmental Protection Agency. Office of Testing and Evaluation. Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules. 40 CFR Part 772. Standard for Development of Test Data. Subpart D. FEDERAL REGISTER. Vol. 44, No.91. Pp. 27350-27362.

(11) Weingand K, Brown G, Hall R et al. (1996). Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies. *Fundam. & Appl. Toxicol.* 29:198-201.

(12) World Health Organization. Part I. Environmental Health Criteria 6, Principles and Methods for Evaluating the Toxicity of Chemicals. (Geneva: World Health Organization, 1978).

(13) World Health Organization. Guidelines for Evaluation of Drugs for Use in Man, WHO Technical Report Series No. 563.(Geneva: World Health Organization, 1975).

(14) World Health Organization. Principles for Pre-Clinical Testing of Drug Safety, WHO Technical Report Series No. 341.(Geneva: WHO, 1966).